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THE PROGNOSTIC VALUE OF MICROVASCULAR DENSITY IN LUNG CARCINOMAS

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Abstract: As one of the first quantifiable methods used in the study of angiogenesis, microvascualar density was performed to all currently known tumors, including lung cancer. There are still numerous controversies regarding the prognostic value of microvascular density. For these reasons, the aim of this study was to investigate the morphology and the density of blood vessels in different pathological types of lung carcinomas using CD34 as antibody. We have included in our study 42 patients. In small cell lung carcinomas, CD34-positive vessels were significantly less in comparison with adenocarcinomas. The peritumoral vessels were significantly more numerous than the intratumoral, excepting for large cell lung carcinomas. The lowest values of microvascular density were found for small cell lung carcinomas. We found statistically significant correlation between microvascular density, tumor stage ($p<0.00021$) and the degree of differentiation ($p<0.0032$). Although MVD can be a useful prognostic element, there are enough data to support its role for assessing efficiency of anti-angiogenic therapy.
Keyword: angiogenesis, microvascular density, lung carcinomas, prognosis.

INTRODUCTION

The growth and progression of malignant tumors is strictly dependent on the vascularisation. No tumor grows beyond a size of 2-3 mm in diameter in the absence of blood vessels. This experimental and clinical phenomenon was demonstrated by Folkman et al. (1971) with almost four decades ago. Besides host vessels co-opting, it has been shown that in the natural evolution of carcinogenesis are form permanently numerous blood vessels through the process known as angiogenesis. Angiogenesis is defined as the formation of new blood vessels from pre-existing ones under the influence of specific vascular endothelial growth factors produced by tumor and inflammatory cells of the host.

Angiogenesis is not a characteristic of malignant solid tumors and appears as an early event during carcinogenesis. This approach was demonstrated in particular for tumors from carcinomas group, such as those of the lung. In this was observed that the proliferation of blood vessels is present from the stage of severe dysplasia, and some authors have reported significant increase in the blood vessels density from the hyperplasia stage- (Dazzi et.al. 1999, Fontanini et al. 1999 and Gazdar 2000). As one of the first quantifiable methods used in the study of angiogenesis, microvascular density (MVD) was performed to all currently known tumor, (Weidner 1998, de Jong JS 2000, Iakovlev et al. 2012) including lung cancer (Giatromanolachy et al. 1997, Fontanini et al. 1997, Pastorino et al. 1997, Chandrachud et al. 1997, O'Byme et al. 2000, Liao et al. 2001, Cox et al. 2001).

Even if many data approaches this topic, there are still numerous controversies regarding prognostic value of MVD. These are caused in particular by the fact that the methodology for the application of the method is different from one study to another, specific endothelial antibodies are different, some authors prefer the method of "hot spot", and others prefer reporting to unit area, that why major differences have been published for MVD even for the same types of tumors.

In the squamous cell lung carcinomas, MVD has been evaluated by numerous authors, but the results are controversial in terms of prognostic (Aikawa et al. 1999, Schor et al. 1998, Ozbudak et al. 2009).

For these reasons, the aim of this study was to investigate the morphology, and the density of blood vessels in different pathological types of lung carcinomas using CD 34 as antibody.

MATERIAL AND METHODS

We included in our study 42 biopsies from patients with different types of lung carcinomas. Specimens were fixed in buffer formalin and paraffin embedded. Five μm thick step sections were performed from each case. Slides from each case were stained with haematoxylin-eosin method for the pathologic diagnosis. Slides were dewaxed and rehydrated, treated by heat-induced epitope retrieval in citrate buffer pH6 for 30' (with PT link module, Dako Cytomation, Denmark) and endogenous peroxidase was blocked with hydrogen peroxide 3%. It was followed by incubation with primary antibody, CD34 (monoclonal mouse anti- human, QBEnd 10clone, dilution 1:25, Dako Cytomation, Denmark) for 30 minutes at room temperature. The LSAB+- HRP working system was used, followed by visualization with 3, 3 diamino-benzidine dyhidrochloride. Nuclei were stained with Lillie's modified haemotoxylin (Dako, Glostrup, Denmark). The full immunohistochemical

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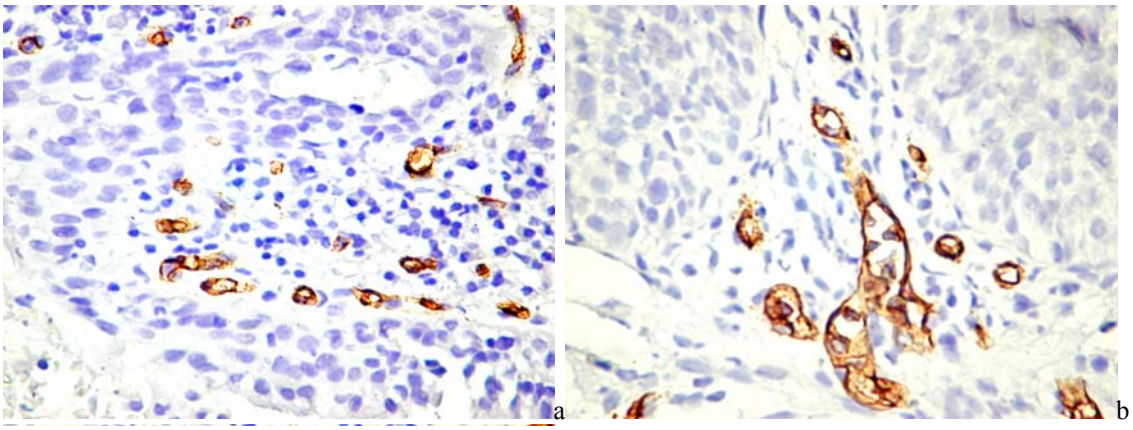
procedure was performed with DakoAutostainer Plus (DakoCytomation). Image acquisition and analysis were performed using Nikon Eclipse E 600 microscope and Lucia G software for microscopic image analysis. Statistical analysis was performed with SPSS13.0 soft, and included Chi square and Student tests, $p<0.05$ being considered as significant. The observations were performed on the sections stained with anti-CD34, based on the fact that in the lung tumor areas and tumor stroma, CD34 selectively identifies only endothelial cells, which facilitates the counting of blood vessels. The assessment was conducted for intratumoral and peritumoral areas, choosing three fields at low magnification with maximum vascular density, by the method known as hot-spot Weidner et al. (1992). The arithmetic mean of the three fields was the outcome of the case. The intratumoral area was defined as the area occupied on the section strictly by tumor cells. The vessels were counted only if they have been found inside the tumor cell area. The data obtained were reported compared to pathological types of lung cancer included in the study and apparently normal lung, adjacent to the tumor.

RESULTS

The examination of sections stained with CD34 revealed the presence of positive blood vessels. The final reaction product was identified in brown in the cytoplasm of endothelial cells (figure 1a). By structure and their disposal, blood vessels showed some morphological features. Thus, in preinvasive lesions, the blood vessels had about the same dimensions, an aspect more relevant in the vicinity of epithelial proliferation. The vessels of peritumoral and intratumoral areas were different in size, irregular as shape, with numerous branches. However, between peritumoral and intratumoral vessels we found some significant differences.

The tumor blood vessels from cases with squamous cell lung carcinomas were identified by intense immunostaining of the endothelium. The vessels are dispose among the tumor cells, distributed relatively uniform throughout the tumor area (figure 1b). We found a great variability in terms of their morphology: variable dimensions, irregular and narrow lumen, intense branching character. Besides blood vessels in the tumor and peritumoral areas we identified small and medium polygonal cells, without obvious lumen which were not taken into account in MVD calculating. Also, while evaluating of tumor area we noticed the presence of many structures with lumen, without positive endothelium, which may reflect the presence of lymphatic vessels and/or CD34 negative blood vessels. In the peritumoral area, the vessels presented constantly large lumen, relatively regular shape and were usually surrounded by inflammatory infiltrate (figure 1c). For this reason, we believe that even sensitive, the method based on CD34 can underestimate the real values of MVD. Only occasionally, in the tumor area we noticed vessels with large lumen, with prominent endothelium to the lumen, an aspect which suggest intussusceptions phenomenon (figure 1d). This process is considered to arise from preexisting blood vessels, included in tumor proliferation. We mention that in none of the cases evaluated by us, we identified tumor cells in the lumen of CD34 positive vessels.



a b

Figure1a- Preinvasive injury, ordered subepitelial vessels, CD34 immunostaining, X400.
Figure1b- Tumor area, vessels with irregular profile, CD34 immunostaining, X400.

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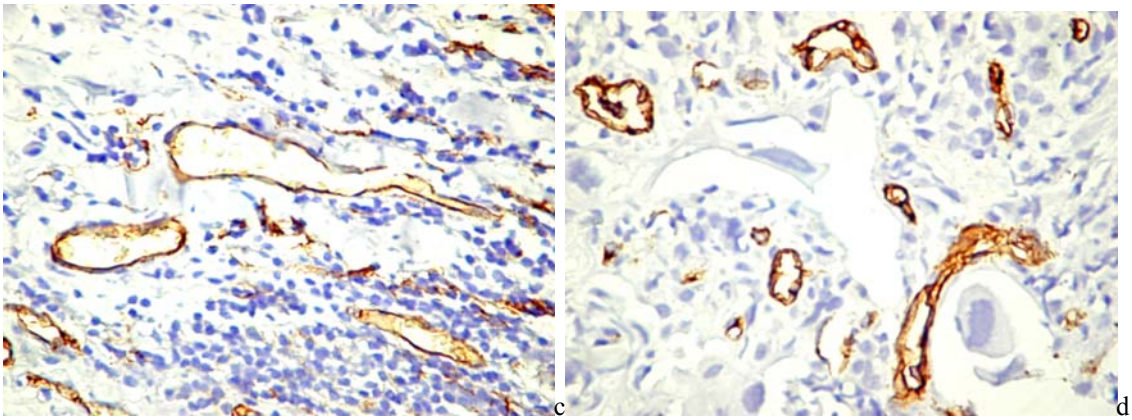

Figure 1c- Peritumoral area, vessels with large lumen, CD34 immunostaining, X100. Figure 1d- intussusception phenomenon, CD34 immunostaining, X400.

In the evaluation of CD34 immunostaining, a few issues have attracted attention. This are related to marker specificity on the one hand and to the vessels morphology of peritumoral and tumor area on the other hand. Frequently, in the vicinity of CD34 positive vessels, we noticed structures with lumen, a very thin wall without content and apparently without perivasculare cells (figure 2a). With the highest probability these structures are lymphatic vessels, whose evaluation is required by lymphatic endothelium specific markers. Simple immunostaining using an endothelial marker can not conclude on the morphologic type of blood vessels, mature or immature nature of bood vessels associated with lung tumors also. Therefore it is necessary to use a double immunostaining method.

The noticed aspects suggest that most of the tumor area vessels are immature and intermediate type (figure 2b). This aspect draws attention to the applicability of the efficient antivasculare therapy in these lesions. An important prognostic factor is the vascular invasion, which has been identified to the 28 of cases included in the study, both in peritumoral (figure 2c), as well as in the intratumoral vessels (figure 2d).

In one case we noticed the endothelial layer discontinuity and prominent of an tumor cells large embolus in the lumen. These issues were more common in the cases of squamous cell lung carcinomas and rare in small cells lung carcinomas.

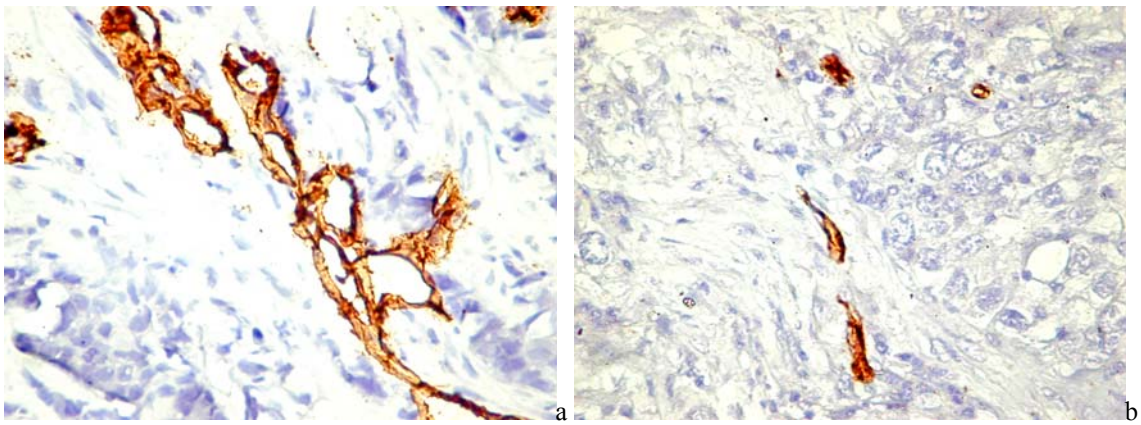

Figure 2a- irregular, crowded vessels, without perivasculare cells, CD34 immunostaining, X400.
Figure2b- Immature vessels, CD34 immunostaining, X400.

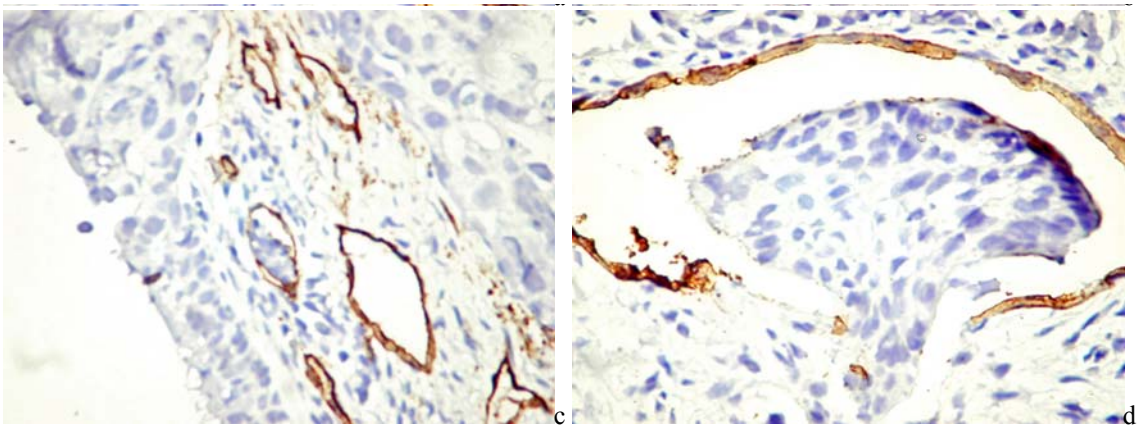

Figure2c- An embolus in a vessel of peritumoral area, CD34 immunostaining, X400. Figure 2d- An embolus in a intratumoral vessel, CD34 immunostaining, X400

MVD values were numerically different from one case to another, and even to the same case in the different areas of the tumor. In some areas, numerous vessels were found (figure 3a). In other tumors, such as small cell lung carcinomas, CD34-positive vessels were significantly less (figure 3b). In lung adenocarcinomas, the vessels were constantly numerous, immature as type and with small dimensions (Figure 3c). Occasionally we noticed the vessels agglomeration, with tendency to form glomeruloid vascular structures (Figure 3d).

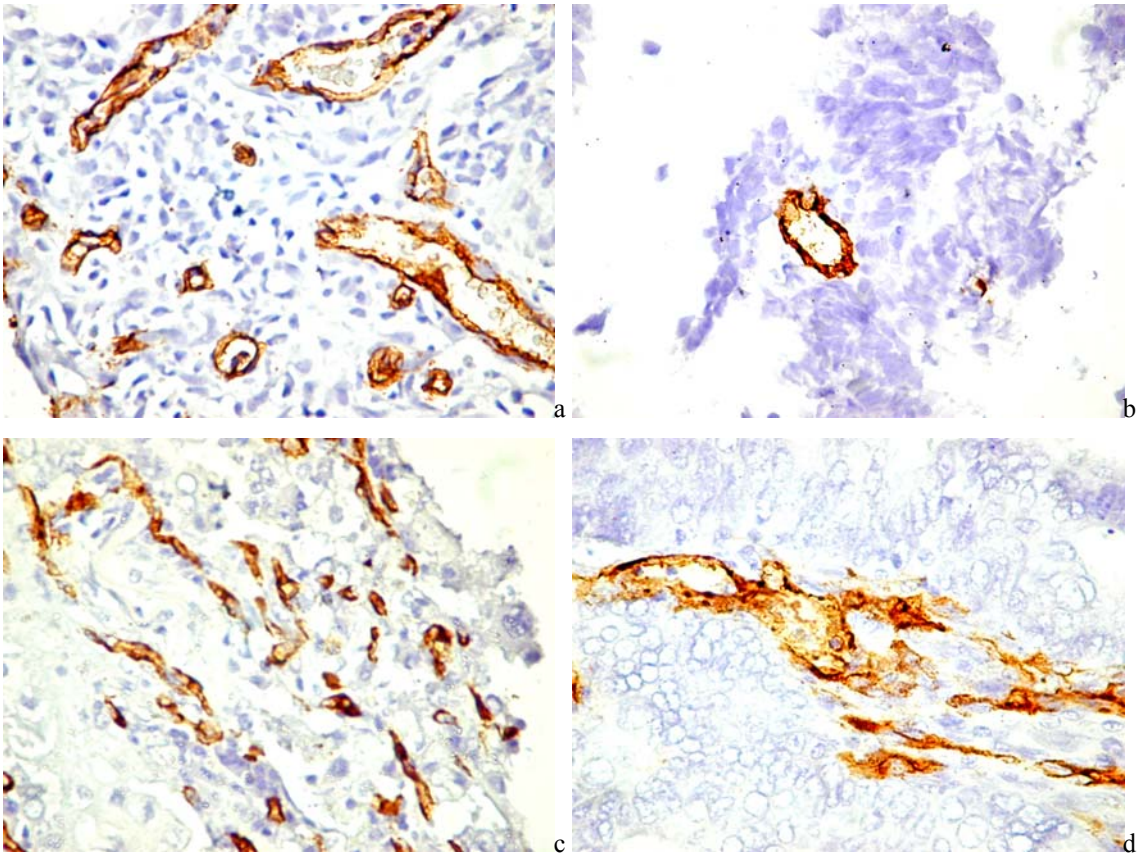

Figure 3a- Numerous vessels with large lumen, CD34 immunostaining, X400. Figure 3b- Rare vessels in small cell lung carcinoma, CD34 immunostaining, X400. Figure3c- a case of lung adenocarcinoma, a high number of vessels in tumor area, CD34 immunostaining, X400. Figure 3d- Glomeruloid vasculare structure, CD34 immunostaining, X 400.

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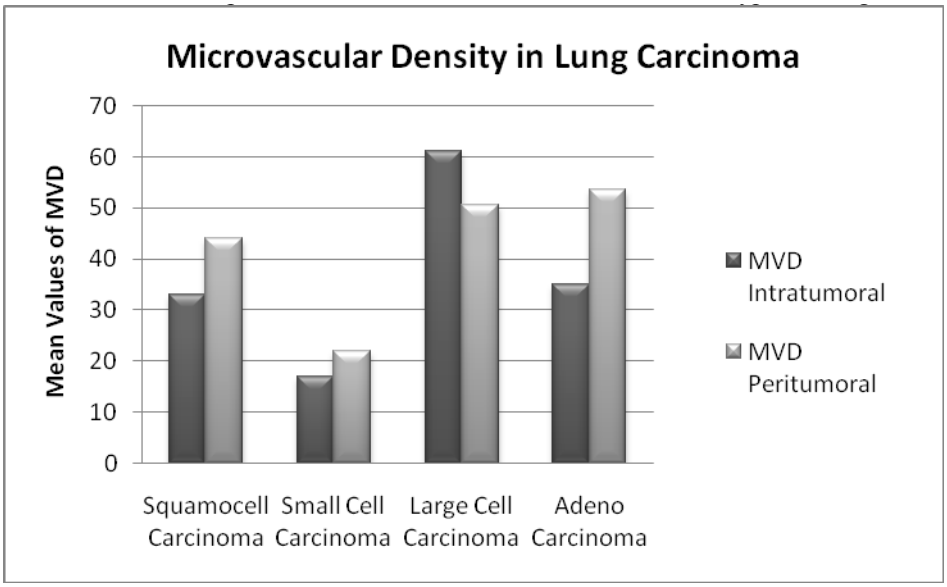
The numerical values of MVD reported to histophatological types of lung carcinomas are presented in Table 1 and Graph no. 1.

Table No.1- The relationship between MVD values and pathological types in lung carcinomas

Pathological type	Intratumoral MVD			Peritumoral MVD		
	Max	Min	Medie	Max	Min	Medie
Squamous cell lung carcinomas	12	54	33	24	64	44
Small cell lung carcinomas	11	23	17	17	27	22
Large cell lung carcinomas	41	81	61	44	57	50.5
Adenocarcinomas	14	56	35	21	86	53.5

From the chart below, it can see that the peritumoral vessels were more numerous than the intratumoral, except for large cell lung carcinomas. Somehow surprisingly, the lowest values of MVD were obtained for small cell lung carcinomas, a tumor recognized for its local and systemic aggressiveness.



Graphic No.1- MVD values in peritumoral and intratumoral areas of different types of lung carcinomas

We found statistically significant correlation between MVD, tumor stage ($p<0.00021$) and the degree of differentiation ($p<0.0032$). No statistical correlation with the age of the patients ($p =0.33$) and the pathological type ($p < 0.24$) was noticed. These results allow us to affirm that the MVD is a useful indicator for local tumor progression, but only partially explains the angiogenic behavior and distant dissemination of neoplastic cells in lung carcinomas.

DISCUSSION

Tumor angiogenesis is an essential requirement for the development, progression, and metastasis of malignant tumors (Hanahan 1996). Immunohistochemical staining measurements of angiogenesis with antibodies to F VIII realted antigen, CD31, CD34, or CD105 can be used to determine microvessel density (MVD), an important prognostic factor that is independent of other known prognostic variables in several cancer types, including lung cancer. Whereas some reports have found that MVD is an important prognostic factor in lung cancer (Giatromanolaki et al. 1997, Fontanini et al. 1997,

O'Byme et al. 2000, Cox et al. 2001), some reports have failed to do so (Pastorino et al. 1997, Chandrachud et al. 1997, Liao et al. 2001). Therefore, although MVD estimation has promising prognostic prospects, a consensus as to whether MVD is a prognostic marker in lung cancer has yet to be reached.

Refere to possible correlations between MVD and tumor properties, (Ge et al. 2000) noticed that MVD was closely related to the size of tumor, lymph node status, TNM stages, grade of cell differentiation, but not to the histological classification, site of cancer, age and sex. We found also a significant correlation between MVD, tumor stage, differentiation grade, but not with age and pathological type.

Carlini et al. (2010) showed that both mast cell and microvessel density were higher in the peritumoral than intratumoral area in non-small cell lung carcinomas. Similar aspect was found by us, a high number of vessels in the peritumoral area in non-small cell lung carcinomas, except large cell lung carcinomas.

Maeda et al. (2011) examined the association between the number of circulating endothelial progenitor cells (EPCs), intratumoral microvessels density, both of which may be markers for neovascularization, and lung cancer histological types, particularly adenocarcinoma histological subtypes. They found no statistically significant differences in the number of EPCs and the MVD between the adenocarcinomas and the squamous cell carcinomas. Among the adenocarcinoma histological subtypes, a higher number of EPCs and MVD were found significantly more frequently in solid than in nonsolid adenocarcinomas. These patients can be the best candidates for anti-angiogenic therapies. We found also almost similar mean values of vessels for squamous cell carcinomas and lung adenocarcinomas

Zhao et al. (2012) demonstrated that there are two major types of microvessels in lung cancer vasculature. The MVD of undifferentiated vessels is a favorable predictor for patients with NSCLC treated with a chemotherapy regimen plus Bevacizumab, with a higher MVD value correlating with better treatment response. Further studies are needed to verify the predictive role of MVD in treatment of NSCLC with Bevacizumab.

Our results confirm the literature data, which signals that MVD is not necessarily an indicator of tumor angiogenesis, it reflect only the presence of a number of vessels at a time. In essence, MVD reflects intercapillary distance, which has a great variability from one tumor to another. Under this specifications, MVD can not be an effective parameter for assessing anti-angiogenic therapy.

CONCLUSIONS

Our observations made on vessels associated with lung carcinomas reveals that CD34 is a marker with high specificity and sensitivity, useful for calculating the MVD in the tumor lesions. MVD is significantly statistical correlated to the degree of differentiation and tumor stage, but not with histopathological type in lung carcinomas.

CONTRIBUTION NOTE

All authors have an equal contribution to this work.

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REFERENCES

Gazdar AF, Minna JD, Angiogenesis and the Multistage Development of Lung Cancers. Clin Cancer Res., 6(5), 1611-1612, 2000.

Aikawa H, Takahashi H, Fujimura S, Sato M, Endo C, Sakurada A, Kondo T, Tanita T, Matsumura Y, Ono S, Saito Y, Sagawa M, Immunohistochemical study on tumor angiogenic factors in non-small cell lung cancer. Anticancer Res., 19(5B), 4305-4309, 1999.

Carlini MJ, Dalurzo MC, Lastiri JM, Smith DE, Vasallo BC, Puricelli LI, Lauría de Cidre LS, Mast cell phenotypes and microvessels in non–small cell lung cancer and its prognostic significance. Hum Pathol., 41(5), 697-705, 2010.

Cox G, Jones JL, Andi A, Waller DA, O'Byrne KJ, A biological staging model for operable non-small cell lung cancer. Thorax, 56*,* 561*–566, 2001.*

Chandrachud LM, Pendleton N, Chisholm DM, Horan MA, Schor AM, Relationship between vascularity, age and survival in non-small-cell lung cancer. Br J Cancer,76*,* 1367*–1375, 1997.*

Dazzi C, Cariello A, Maioli P, Solaini L, Scarpi E, Rosti G, Lanzanova G, Marangolo M, Prognostic and predictive value of intratumoral microvessels density in operable non-small-cell lung cancer. Lung Cancer*,* 24*,* 81-*88,* 1999.

de Jong JS, van Diest PJ, Baak JP, Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer. Histopathology, 36(4), 306-312, 2000.

Folkman J, Merler E, Abernathy C, Williams G, Isolation of a tumor factor responsible for angiogenesis. J Exp Med., 133, 275-288, 1971.

Fontanini G, Calcinai A, Boldrini L, Lucchi M, Mussi A, Angeletti CA, Cagno C, Tognetti MA, Basolo F, Modulation of neoangiogenesis in bronchial preneoplastic lesions. Oncol. Rep.*,* 6*,* 813-*817,* 1999.

Fontanini G, Lucchi M, Vignati S, et al., Angiogenesis as a prognostic indicator of survival in non-small-cell lung carcinoma: a prospective study. J Natl Cancer Inst., 89*,* 881*–886, 1997.*

Ge G, Zhou Q, Zhang S, Liu L, Chen J, Cheng N, Qin J, Li L, Detection of micro-vascular density(MVD) and its clinical significance in lung cancer. Zhongguo Fei Ai Za Zhi., 3(4), 284-287, 2000.

Giatromanolaki A, Koukourakis MI, Theodossiou D, et al., Comparative evaluation of angiogenesis assessment with anti-factor-VIII and anti-CD31 immunostaining in non-small cell lung cancer. Clin Cancer Res., 3*,* 2485*–2492, 1997.*

.Hanahan D, Folkman J., Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell., 86, 353–364, 1996.

Iakovlev V, Gabril M, Dubinski W, Scorilas A, Youssef M, Faragalla H, Kovacs K, Rotondo F, Metias S, Arsanious A, Plotkin A, Girgis A, Streutker CJ, Microvascular Density as an Independent Predictor of Clinical Outcome in Renal Cell Carcinoma An Automated Image Analysis Study. Laboratory Investigation, 92(1), 46-56, 2012.

Liao M, Wang H, Lin Z, Feng J, Zhu D, Vascular endothelial growth factor and other biological predictors related to the postoperative survival rate on non-small cell lung cancer. Lung Cancer, 33*,* 125*–132, 2001.*

Maeda R, Ishii G, Ito M, Hishida T, Yoshida J, Nishimura M, Haga H, Nagai K, Ochiai A, Number of Circulating Endothelial Progenitor Cells and Intratumoral Microvessel Density in Non-small Cell Lung Cancer Patients: Differences in Angiogenic Status between Adenocarcinoma Histologic Subtypes. J Thorac Oncol., Epub ahead of print Dec 22, 2011.

O'Byrne KJ, Koukourakis MI, Giatromanolaki A, et al. Vascular endothelial growth factor, platelet-derived endothelial cell growth factor and angiogenesis in non-small-cell lung cancer. Br J Cancer, 82*,* 1427*–1432, 2000.*

Ozbudak IH, Ozbilim G, Kucukosmanoglu I, Dertsiz L, Demircan A, Vascular endothelial growth factor expression and neovascularization in non--small cell lung carcinoma. Int J Surg Pathol., 17(5), 390-395, 2009.

Pastorino U, Andreola S, Tagliabue E, et al., Immunocytochemical markers in stage I lung cancer: relevance to prognosis. J Clin Oncol., 15*,* 2858*–2865, 1997.*

Schor AM, Pazouki S, Morris J, Smither RL, Chandrachud LM, Pendleton N, Heterogeneity in microvascular density in lung tumours: comparison with normal bronchus. Br J Cancer., 77(6), 946–951, 1998.

Weidner N., Tumoral vascularity as a prognostic factor in cancer patients: the evidence continues to grow. J Pathol.,184, 119–122, 1998.

Zhao YY, Xue C, Jiang W, Zhao HY, Huang Y, Feenstra K, Resau JH, Qian CN, Zhang L, Predictive value of intratumoral microvascular density in patients with advanced non-small cell lung cancer receiving chemotherapy plus bevacizumab. J Thorac Oncol., 7(1), 71-75, 2012.